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(54) Title: ANTIGENS OF *PLASMODIUM FALCIPARUM*

(57) Abstract

DNA molecules comprising artificially constructed polynucleotide sequences substantially corresponding to all or a portion of the base sequence coding for an antigen of *Plasmodium falciparum* selected from the group consisting of the RESA antigen, the FIRA antigen, and other antigens of *P.falciparum* cross-reactive therewith. Such DNA molecules are capable of being expressed as polypeptide(s). Synthetic peptides or polypeptides displaying the antigenicity of all or a portion of the RESA or FIRA antigens of *P.falciparum*. Compositions for stimulating immune responses against *P.falciparum* antigens in a mammal, comprising at least one polypeptide displaying the antigenicity of the RESA or FIRA antigens of *P. falciparum*, together with a pharmaceutically acceptable carrier therefor.

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ANTIGENS OF PLASMODIUM FALCIPARUM

This invention relates to synthetic peptides and polypeptides which have antigenicity suitable for providing protective immunity against Plasmodium falciparum infections, and to methods for the production thereof.

Immunity to Plasmodium falciparum, the protozoan causing the most severe form of human malaria, is acquired only after extensive exposure over a number of years. A large number of P.falciparum polypeptides are natural immunogens in man but it is by no means clear how many are important in protective immunity. Many antigens may have no such role, and indeed it is possible that some are counterproductive, perhaps because collectively they overload the immune system. Antigenic diversity among different strains of the parasite may also play a significant role in the process of immune evasion as a number of P.falciparum antigens that are strain-specific have been identified.

Recently, molecular cloning techniques have facilitated the analysis of individual polypeptide antigens of P.falciparum (1). Many cDNA clones encoding these antigens have been isolated by screening Escherichia coli colonies that express the cloned sequences with human antibodies. The production and

screening of these clones is described in detail in International Patent Specification No. PCT/AU84/00016.

One such antigen has been located at the surface of erythrocytes infected with the immature ring stage of P.falciparum and hence has been designated the Ring-infected Erythrocyte Surface Antigen (RESA). Because of this exposed location, it appears to be a likely target for immune attack. RESA shows the structural peculiarity that has now been found in a number of Plasmodium antigens, namely multiple tandem repeats of oligopeptides (2-6).

Studies by hybridization and by immunofluorescence suggest that RESA from the Papua New Guinea isolate FC27, may be conserved in a wide range of P.falciparum isolates, including strain NF7 from Ghana. The relationship between RESA cDNA clones from two different strains of P.falciparum has therefore been studied by immunological and sequencing methods. Antibodies that reacted with RESA from strain FC27 of Papua New Guinea were present in patients from Africa and conversely, antibodies that reacted with RESA from strain NF7 were present in patients from Papua New Guinea. From the complete nucleotide sequences of eight cDNA clones encoding portions of RESA from P.falciparum strains FC27 and NF7, it is concluded that the RESA polypeptides from the two strains are closely homologous. The sequencing of these cDNA clones identified in the RESA polypeptide two separate blocks of tandem sequence repeats. One block of repeats, located at the C terminus of RESA in FC27, contains four different but related acidic sequences of eight, four, four and three amino acids. Approximately 600 bases 5'

is a second block of repeats encoding related amino acid sequences which are also rich in acidic amino acids. Consistent with the sequence relationships, the two blocks of repeats have been shown to encode cross-reacting antigenic epitopes.

Immunoblots on the antigens of synchronously growing parasites separated on SDS-PAGE suggested that RESA is synthesized in the mature trophozoite as a Mr 210,000 protein which is processed to the Mr 155,000 form found bound to the membrane of erythrocytes infected with ring stage parasites. The more recent finding that the Mr 210,000 protein does not react with several anti-RESA monoclonal antibodies and anti-RESA peptide antibodies suggests that the Mr 210,000 protein is a cross-reacting antigen and not a precursor of the Mr 155,000 RESA molecule.

The Mr 155,000 polypeptide in merozoites is soluble in the non-ionic detergent Triton X-100 but after transfer to the membrane of the ring-infected erythrocyte it is largely Triton-insoluble. Thus, it seems likely that RESA interacts with the erythrocyte cytoskeleton. Whether RESA penetrates the membrane lipid bilayer is not yet clear, but an important clue may come from the complete sequence of the RESA gene which has now been determined. From this, it is deduced that RESA contains two exons separated by a short intervening sequence (Figure 2). Exon 1 commences with a hydrophobic sequence typical of signal peptides on secreted polypeptides in many organisms. Following this, there is a hydrophilic sequence of approximately 36 amino acid residues and then a second hydrophobic stretch, of 14 residues. 202 bases further downstream

exon 2 commences with a 16 amino acid non-charged sequence and then continues with a highly charged region. The hydrophobic sequence generated by excision of the intron is typical of membrane-anchor segments in a number of eukaryotic genes.

As a result of work leading to the present invention, described in detail below, it has been shown on the basis of sequence, hybridization and immunological data that it is likely that RESA is highly conserved in most or all strains of P.falciparum. In addition, as the repetitive structure and the location of RESA at the surface of ring infected erythrocytes are properties highly suited for sensitive detection by such procedures as indirect immunofluorescence, the high degree of immunological similarity of RESA in different strains suggest that RESA is a molecule well suited for immunodiagnostic purposes.

Another antigen detected as a result of its cloning and expression in E.coli has been designated the Falciparum Interspersed Repeat Antigen (FIRA) (6). Like some other repetitive antigens FIRA contains a structural unit bearing repeats of a short unit flanked by a highly charged region. However, this entire structural unit is itself repeated several times within the antigen.

The corresponding cDNA clone expressing FIRA in Escherichia coli reacted in an in situ colony assay with sera from up to ~93% of people living in an area endemic for P.falciparum. Human antibodies affinity-purified on immobilized lysates of the corresponding cDNA clone identified the corresponding

parasite antigen as a polypeptide of Mr >300,000. It was present in schizonts and also in ring-stage trophozoites, where a speckled immunofluorescence pattern suggested an association with the erythrocyte. Its mRNA was enriched in merozoites, a distinctive property shared by RESA which is located on the surface of ring-infected erythrocytes and it is encoded by a single gene with a number of allelic variants. The complete nucleotide sequence of the cDNA clone revealed a structural unit comprised of 13 hexapeptide repeats flanked by a highly charged region containing both acidic and basic amino acids. This structural unit is itself repeated, so that blocks of repeats and charged units are interspersed along the molecule. The sequence within the repeats vary much more extensively than those in the charged units.

The sequence of a chromosomal FIRA clone demonstrates that the FIRA gene is organised in a manner analogous to that of RESA (Figure 8). It contains a short 5' exon, a much longer 3' exon and a hydrophobic segment at the boundary of the two exons. As with RESA, the repeats in FIRA are restricted to the 3' exon only.

According to the present invention, there is provided a DNA molecule comprising a nucleotide sequence substantially corresponding to all or a portion of the base sequence coding for an antigen of P.falciparum selected from the group consisting of the Ring-infected Erythrocyte Surface Antigen (RESA), the Falciparum Interspersed Repeat Antigen (FIRA), and other antigens of P.falciparum cross-reactive therewith. In particular, there is provided a DNA molecule comprising a nucleotide sequence characterised by at least a

portion thereof comprising all or a portion of the base sequence shown in Figure 1 or Figure 7. Such a nucleotide sequence codes for a polypeptide comprising at least a portion which corresponds to the amino acid sequence of RESA or FIRA.

As noted above, and set out in greater detail in Figure 1 and 7, the amino acid sequences of RESA and FIRA consist of repeat units and flanking non-repeat peptide units. Accordingly, the base sequences referred to above may code for polypeptides corresponding to one or more of these repeat and/or flanking units, or to polypeptides corresponding to combinations of these repeat and/or flanking units.

The present invention also extends to synthetic peptides or polypeptides displaying the antigenicity of all or a portion of an antigen selected from the group consisting of the RESA antigen, the FIRA antigen, and other antigens of P.falciparum which are cross-reactive therewith, as well as to compositions for stimulating immune responses against P.falciparum in a mammal, which compositions comprise at least one synthetic peptide or polypeptide as described above, together with a pharmaceutically acceptable carrier therefor. The synthetic peptides or polypeptides according to this aspect of the invention may be prepared by expression in a host cell containing a recombinant DNA molecule which comprises a nucleotide sequence as broadly described above operatively linked to an expression control sequence, or a recombinant DNA cloning vehicle or vector containing such a recombinant DNA molecule. The synthetic peptide or polypeptide so expressed may be a fusion polypeptide comprising a

portion displaying the antigenicity of all or a portion of RESA or FIRA or other cross-reactive antigen, and an additional polypeptide coded for by the DNA of the recombinant DNA molecule fused thereto. Alternatively, the synthetic peptides or polypeptides may be produced by chemical means, such as by the well-known Merrifield solid-phase synthesis procedure.

Further details of the present invention will be apparent from the detailed description hereunder, and from the accompanying Figures. In the Figures:

Figure 1 shows the nucleotide sequence and predicted amino acid sequence of RESA. The nucleotide sequence was determined by the dideoxy procedure (8).

Figure 2 shows the structure of the RESA gene, as deduced from the sequence given in Figure 1. The 5' and 3' exons are indicated.

Figure 3 shows:

A. Western blot of asynchronous cultures of two isolates of P.falciparum lysed in electrophoresis sample buffer and probed with anti-RESA antibodies. B.& C. Western blots of P.falciparum (1) ring stages, (2) mature trophozoites, (3) schizonts, and (4) merozoites using affinity-purified human antibodies to RESA. (B) Antigens extracted in Triton X-100. (C) Antigens insoluble in Triton X-100 but soluble in electrophoresis sample buffer. Radioactive molecular weight markers were obtained from Amersham Internat., Buckinghamshire, England

and were myosin (200 Kdaltons), phosphorylase-b (93 Kdaltons) and bovine serum albumin (69 Kdaltons).

Figure 4 is an immunoelectronmicrograph showing the location of RESA (→) in small dense vesicles presumably micronemes within the developing merozoites in a schizont, detected with rabbit anti-RESA and protein A gold. The rhoptries (R) are unlabelled. (x 41,700; inset x 73,000).

Figure 5 is an immunoelectronmicrograph showing a section of a ring-infected erythrocyte reacted with rabbit anti-RESA. Also shown is part of an uninfected erythrocyte.

Figure 6 is a Western blot of ring-stage infected erythrocytes digested with chymotrypsin (20µg/ml) for 0 min. (1), 20 min. (2) and 60 min. (3). Subsequent to enzyme digestion the intact erythrocytes were washed, lysed in electrophoresis sample buffer, electrophoresed on a 10% SDS-polyacrylamide gel and then electrophoretically transferred to nitrocellulose. The nitrocellulose filters were then probed with rabbit anti-RESA at a dilution of 1:500. Molecular weights are indicated in Kdaltons, and correspond to RESA (155Kd), β-galactosidase (116Kd) and phosphorylase-b (93Kd).

Figure 7 shows the nucleotide sequence and predicted amino acid sequence of the FIRA gene. The nucleotide sequence was determined by the dideoxy procedure (8). The EcoR1 linker ligated to the 3' end during construction of the library was absent and so the sequence is incomplete at the 3' end, perhaps due to a deletion.

Figure 8 shows the structure of the FIRA gene as deduced from the sequence given in Figure 7.

Figure 9 shows immunoassays (A & B) and Western blots (C & D) with human antibodies affinity-purified from a serum pool derived from individuals exposed to malaria. In A and C the antibodies were purified on a FIRA-Sepharose absorbent whereas in B & D the antibodies were purified on an λamp3-Sepharose absorbent. The P.falciparum isolates in C and D were: 1, FC27 from Papua New Guinea; 2, K1 from Thailand; and 3, NF7 from Ghana.

Figure 10 shows affinity purified anti-FIRA antibodies assayed by solid-phase ELISA using microtitre plates coated with purified fusion polypeptides (2µg/ml) corresponding to: ○, a fragment of FIRA; □, 5' repeat by RESA; △, 3' repeat of RESA.

DETAILED DESCRIPTION OF THE INVENTION

MATERIALS AND METHODS

P.falciparum isolates

Isolates FCQ27/PNG (FC27), IMR143/PNG (IMR143), IMR144/PNG (IMR144) and MAD71/PNG (MAD71) were obtained through collaboration with the Papua New Guinea Institute of Medical Research. NF7, originating from Ghana, and K1 originating from Thailand were obtained from D.Walliker, Edinburgh University.

Colony Immunoassays

Replicas of arrays of antigen-positive clones were grown overnight at 30°C, induced at 38°C, and lysed (7).

Sera were absorbed to remove anti-E.coli reactivity, diluted 1:500 at pH 9.6 in 3% bovine serum albumin and finally incubated with ^{125}I protein A from Staphylococcus aureus and autoradiographed overnight (7).

Sera

Sera were obtained with informed consent from individuals from Madang, Papua New Guinea. Some patients presented with acute malaria while in others, asymptomatic parasitemia was detected in the course of routine surveys. Parasitemic individuals were treated with chloroquine. Parental consent was obtained before taking samples from children.

Hybridization experiments

The phage DNA was purified by CsCl-equilibrium density centrifugation, digested with EcoRI, and size-fractionated on a 1% low-melting agarose-gel, recovered by phenol extraction and labelled by nick-translation. 3ml of labelled insert (3×10^5cpm) in 1ml 0.75 M NaCl/0.75 M Na citrate/50% formamide/50µg ml^{-1} salmon sperm DNA/10µg ml^{-1} poly (C)/0.02% Ficoll/0.02% polyvinylpyrollidone/0.02% BSA was hybridized to the array of antigen-positive clones. The inserts were subcloned in pUC-9 (9), purified and then nick-translated as described above and used in Southern blot experiments.

Isolation and sequencing of cloned chromosomal segments

The chromosomal RESA clones were isolated from a λgt10 library, and the EcoR1 inserts subcloned into pUC8. Rsa I, Aha III and Ssp I fragments of the EcoR1 inserts were subcloned into M13mp18 and mp19 vectors,

and sequenced by the dideoxy technique (8). Synthetic primers were also used. The results were processed by the program of Staden (10). The sequence shown consists of the 3.5 Kb chromosomal EcoR1 fragment, joined at the EcoR1 site to that of the cDNA clone Ag 46.

The chromosomal FIRA clone was initially identified as a 6 Kb Aha III fragment in λgt10. This Aha III fragment was subcloned into pUC8. Pvu II and Rsa I fragments were then subcloned into M13mp8 and 9 vectors and sequenced by the dideoxy technique.

Affinity purification of anti-RESA and anti-FIRA antibodies.

Induced cultures (50ml) of clones Ag28, Ag231 and λamp3 were prepared as described previously (5 and 6). The pelleted bacteria were sonicated in 100mM Na phosphate buffer, pH 6.8/10mM dithiothreitol followed by mixing at room temperature with the addition of 1% $NaDodSO_4$. The soluble bacterial proteins were equilibrated with 100mM Na phosphate, pH 6.8/1mM dithiothreitol/0.1% $NaDodSO_4$ by passage through Sephadex G-10 and conjugated to CNBr-activated Sepharose (Pharmacia, Sweden) at room temperature according to the manufacturers instructions.

A pool of human sera collected from individuals living in Papua New Guinea was clarified by centrifugation, diluted with an equal volume of phosphate buffered saline (Pi/NaCl) and preabsorbed on a λamp3-Sepharose absorbent before passage over the Ag28 or Ag231 absorbent. Non-specifically bound proteins were removed by repeated wash cycles of 100mM Na borate/500mM NaCl/0.05% Tween 20, pH 8.5 followed by

Pi/NaCl. Bound antibodies were eluted with 100mM glycine/150mM NaCl, pH 2.6 and immediately adjusted to pH 7.0 with 2M Tris;HCl, pH 8.0.

Western blots

Protein extracts of cultures of P.falciparum were prepared and fractionated on 7.5% polyacrylamide/NaDodSO$_4$ gels. Proteins from the gels were transferred electrophoretically to nitrocellulose, incubated in 5% non-fat milk powder in Pi/NaCl before reaction with affinity purified human antibodies. The filters were incubated with ^{125}I-labelled protein A and autoradiographed.

Immunoelectronmicroscopy

Human antibodies affinity purified on Ag28 and Ag231 immunosorbents, or rabbit antisera raised against the fused polypeptide produced by Ag28 were used in immunoelectronmicroscopy employing the protein A-gold procedure. Samples for immunoelectronmicroscopy were fixed with 0.25% glutaraldehyde (10 min at 25°C), diluted in 50mM NH_4Cl in 0.1M phosphate buffer, pH7.4, and then left in fresh 50mM NH_4Cl in phosphate buffer for 30 min. Cells were then washed twice in phosphate buffer and dehydrated in 70% ethanol before being embedded in L.R. White resin, hard grade (London Resin Co. Ltd., Basingstoke, England). Sections were incubated in 1% bovine serum albumin or ovalbumin in 0.05M phosphate, pH7.4, containing 0.25% Tween-20 (PO_4:Tween) for 5 min. before transfer to a drop of rabbit anti-RESA antiserum (diluted 1:100) or affinity-purified human anti-RESA antibodies in PO_4:Tween for 30-60 min. at room temperature. After being washed in PO_4:Tween the sections were transferred

to protein A-gold (E-Y Laboratories, Inc.) diluted 1:10 in PO_4:Tween for 30-60 min. After further washing, the sections were stained with aqueous uranyl acetate. Isolated merozoites were fixed at 4°C in 0.25% glutaraldehyde for 10 min. and then processed in the same manner as infected cells.

RESULTS - RESA

Isolation of a RESA cDNA clone from FC27

The preparation of the RESA cDNA clones is described in detail in the Examples of International Patent Specification No. PCT/AU84/00016, and incorporated herein by reference.

Identification of the RESA polypeptides

Human antibodies specific for the RESA polypeptides were purified by affinity chromatography. In Western blots the antibodies reacted with a prominent band at Mr 155,000 which, in some experiments, resolved into a closely migrating doublet. A higher molecular weight polypeptide reacting with the anti-RESA antibodies varied in size in different isolates (Figure 3A); it was at Mr 210,000 in isolate FC27. In addition, a smaller molecular weight polypeptide (Mr 80,000) was detected in some antigen preparations (Figure 3A). The abundance of the Mr 210,000 polypeptide was greatest in schizonts (Figure 3B). In contrast, the Mr 155,000 antigen was abundant in the merozoites, rings and trophozoites with small amounts of schizonts (Figure 3, B and C.)

The solubility of RESA in detergents was determined to examine the nature of the interaction between RESA

and the erythrocyte membrane. The Mr 210,000 polypeptide was soluble in solutions of the nonionic detergent Triton X-100, as was the most of the Mr 155,000 polypeptide present in merozoites (Figure 3B). In contrast, the bulk of the Mr 155,000 antigen in rings and other life-cycle stages was insoluble in Triton X-100 but could be solubilised in electrophoresis sample buffer containing SDS and 2-mercaptoethanol (Figure 3, B and C).

When identical immunoblots were probed with monoclonal antibodies raised against the Ag28 fused polypeptide, or antisera raised in mice against RESA synthetic peptides, the Mr 210,000 polypeptide was not detected although the Mr 155,000 polypeptide gave a strong signal. Thus, it appears that the Mr 210,000 polypeptide is another gene product that cross-reacts with RESA and not the initial RESA translation product.

Antibodies against RESA in patients from Africa react with RESA from a Papua New Guinea strain.

Previous studies with mouse antibodies against RESA fused polypeptides expressed in E.coli demonstrated cross reactions with all P.falciparum strains tested, from diverse locations. These RESA cDNA clones were isolated by virtue of their reactivity to sera from Papua New Guinea. To determine whether equivalent antibodies that cross react with RESA from widely differing locations occur in humans exposed to P.falciparum, African sera were tested against a number of cDNA clones expressing portions of RESA, derived from the Papua New Guinea strain FC27. The sera were reacted with an array of 133 independently isolated antigen positive clones, 16 of which encoded RESA, by the

in situ colony immunoassay procedure as described (7). Both African sera reacted with the RESA cDNA clones. The extent of reaction was quite comparable to many of the PNG sera. However, it is important to note that the extent of reaction varies considerably in different PNG sera. The African sera also reacted with a variety of other cDNA clones including cDNA clones that encode FIRA that consists largely of divergent repeats of a hexapeptide sequence. In contrast, they did not react with cDNA clones encoding the strain-specific S-antigen of FC27. Thus RESA polypeptides from geographically diverse areas must share non-reacting epitopes that are natural immunogens in man.

Antigenic determinants of RESA

All RESA cDNA expression clones previously studied immunologically were bounded at the 5' terminus by the internal EcoR1 site. To examine whether any antigenic determinants were located 5' to this site, the large EcoR1 fragment from NF7 AG13 was subcloned into pUC9, randomly fragmented by sonication and the fragments were recloned in λAmp3. To identify clones expressing defined regions of this fragment, the resulting clones were screened by hybridization with 3 different restriction fragments, located 5' to the repeats, spanning the repeats and 3' to the repeats, respectively. Selected clones were then examined for expression of large fused polypeptides, detectable by Coomassie blue staining after polyacrylamide gel electrophoresis of total protein extracts from the cells. Because there are multiple stop codons in all but the correct frame of the sequence, it could be concluded that such clones expressed defined fragments

of RESA, 5' to any fragments that had previously been analysed for antibody binding.

Clones expressing 5' repeats were then examined by in situ colony immunoassays with sera from PNG patients with a history of exposure to P.falciparum. Some clones containing the 5' repeat segment reacted with the sera. It is concluded that there are antigenic determinants that are natural immunogens in man in the 5' RESA repeats, as well as the 3' repeats.

A 36 amino acid peptide corresponding to the sequence from residue 17 to residue 52 in exon 1 of RESA (Figure 1) was synthesised and used to test sera from individuals exposed to malaria for antibodies to this region of RESA. Some individuals had significant levels of antibodies reactive with this peptide as measured in a solid-phase radio-immunoassay. Thus there are naturally immunogenic epitopes in exon 1 of RESA which must be encoded by non-repeat sequences.

Immunogenicity of RESA sequences

RESA/β-galactosidase fused polypeptides were isolated from clones expressing the 3' and 5' repeats of RESA. These proteins were tested for immunogenicity by immunising rabbits with 0.25-0.5mg amounts of antigen together with complete Freund's adjuvant. The rabbits were boosted with similar amounts of antigen in incomplete adjuvant 4-6 weeks later. In each case, antibodies were elicited which reacted with the RESA molecule expressed in P.falciparum growing in vitro.

Three RESA synthetic peptides (Table 1) conjugated to Keyhole Limpet Haemocyanin, were used to immunise

mice and the resulting antisera were assayed against each of the three peptides conjugated to bovine serum albumin, and against fused polypeptides corresponding to the 3' and 5' repeats of RESA and sonicates of infected erythrocytes. All mice immunised with these peptides produced antibodies that were reactive with the homologous peptide and the fused polypeptide containing that sequence. In addition, peptide RESA 3'-2 (EENV x4), induced antibodies that also reacted with the other 3' repeat peptide, RESA 3'-1 (EENVEHDA) which has a 5 amino acid sequence in common. The reverse, however, was not true: anti-RESA 3'-1 antibodies did not react with RESA 3'-2.

When these anti-peptide antisera were assayed on peptide-BSA conjugates there was no apparent cross-reactivity between the 5' and 3' repeats of RESA. However, assaying the same sera on fused polypeptides revealed that the peptides had induced antibodies that reacted with both repeat structures, although the reaction with the heterologous repeat was very weak in comparison to that with the homologous repeat.

The anti-peptide antisera were used to probe Western blots of infected erythrocytes. All of the antisera reacted specifically with the Mr 155,000 RESA polypeptide.

TABLE 1

Sequences and synthetic peptides corresponding to repeats in RESA

Region of RESA	Repeat Sequences	Peptides Synthesized*
3' Repeat	EENVEHDA (5)+	RESA 3'-1 EENVEHDA
	EENA (1)	
	EENV (29)	RESA 3'-2 (EENV)n n ∿4
	EE-V (4)	
	EEYD (3)	
5' Repeat	-EENEEEHTV- (1)	
	DDEHVEEHT-A (1)	
	DDEHVEEPTVA (2)	RESA 5'-1 DDEHVEEPTVAY
	-DEHVEEPTVA (1)	
	-EEHVEEPTVA (1)	
	-EEHVEEP--A (1)	

* The peptides were synthesized by the Merrifield solid-phase method except the RESA 5'-1 peptide was synthesized by the FMOC solid-phase synthesis methodology of Atherton et al (11) on a Kieselguhr KA resin support.

+ The numbers in brackets indicate the number of times the respective sequences occur within the blocks of repeats.

Location of RESA

RESA was detected by immunoelectronmicroscopy at the membrane of erythrocytes infected with ring-stage parasites but not in association with immature parasites within the erythrocyte (Figure 5). In contrast, the membranes of erythrocytes containing mature parasites were not labelled, but gold particles were associated with electron-dense organelles presumed to be micronemes within the parasite cytoplasm (Figure 4). Control antibodies to S antigens did not react with merozoites or the erythrocyte membrane.

The labelling of merozoites was clearly internal, with no indication of specific labelling of the merozoite surface. Labelling occurred in clusters away from the nucleus and occasionally over a rhoptry. In other merozoites, gold particles were more dispersed but were located near the rhoptries, which were particle-free. Similar distributions of gold were observed with both affinity-purified human antibodies and rabbit antibodies raised against the cloned antigen, although higher background labelling was evident with the affinity-purified human antibodies. The specificity of the observed patterns of labelling was demonstrated by the different patterns, or by the lack of labelling when the same procedures were used with affinity-purified human antibodies or rabbit antisera to other cloned P.falciparum antigens (e.g. S antigen).

The location of RESA was further examined by studying its accessability to attack by proteolytic enzymes. When intact erythrocytes infected with ring-stage parasites (approximately 5% parasitaemia) were treated with chymotrypsin or trypsin, the

Mr 155,000 polypeptide was partially cleaved at a limited number of sites generating two main fragments which like the intact molecule reacted with anti-RESA antibodies (Figure 6). This result indicates that at least part of the RESA molecule is exposed on the external surface of the ring-infected erythrocyte.

Inhibition of parasite growth in vitro

Asynchronous cultures of P.falciparum were cultured for 48 hours in the presence of affinity-purified human anti-RESA antibodies. The degree of inhibition was variable with typical results showing 20-40% inhibition compared with control cultures.

RESULTS - FIRA

cDNA clones expressing FIRA

FIRA cDNA clones reacted with up to 93% of a set of more than 100 PNG sera from 65 individuals, varying in clinical status. Further, they gave the most intense signals with a majority of the sera, although many sera reacted strongly with additional clones.

FIRA is encoded by a single polymorphic gene

Chromosomal DNA from 5 P.falciparum isolates (FC27, IMR143, IMR144, MAD71 from Papua New Guinea, and NF7 from Ghana) was restricted with EcoRI, AhaIII and RsaI and analysed by Southern blotting. In each isolate, a single very large (>20 kb) EcoRI fragment hybridized (data not shown). In the AhaIII and RsaI digests, smaller single fragments of varying sizes hybridized, revealing that the FIRA gene was polymorphic and present in each isolate investigated. The different fragment sizes most likely represent different alleles of the

FIRA gene. As at least three different alleles were detected in only 5 different isolates, the total number of alleles is presumably very large. The single fragment size in each isolate is in accord with a haploid genome in blood stage Plasmodium.

Identification of the FIRA polypeptide

Human antibodies specific for the FIRA polypeptide (Fig. 9) were purified by affinity chromatography. In Western blots the antibodies reacted strongly with a P.falciparum polypeptide of very large apparent size, nominally of Mr >300,000, that was present in each isolate (Fig.9C). Although there were no accurate size markers in this extreme range, the mobility of FIRA was considerably less than that of the Mr 200,000 S antigen of FC27. Isolate differences in the sizes of FIRA polypeptides that might be expected to correlate with the slight differences in size of the DNA fragments could not be detected (Fig. 9C). The antibodies also reacted weakly and variably with a number of smaller polypeptides (Fig. 9C), presumably proteolytic cleavage products of the Mr >300,000 molecule. The control antibodies, purified from the same serum on a vector absorbent did not react (Fig.9D). Further, antibodies purified from the same serum on absorbents from other antigen-positive clones reacted specifically with other polypeptides, not the Mr >300,000 polypeptide (data not shown).

It is concluded that FIRA is a very large polypeptide that is expressed in each isolate of P.falciparum tested, and that antibodies to the allele of FIRA expressed by FC27 cross-react with the alleles expressed by K1 and NF7.

Location and stage specificity of FIRA and its mRNA

The affinity-purified human antibodies and serum from mice immunized with clone Ag231 or members of the Ag231 family reacted with mature parasites (containing pigment) and also with cells containing immature (ring-form) parasites. The fluorescence over ring-infected cells was uneven and apparently distributed beyond the limits of the parasite. Hence it is likely that FIRA is external to the parasite, although no staining of the erythrocyte surface was detected when the antibodies were reacted in suspension with non-fixed parasitized cells or with lightly glutaraldehyde-fixed and air-dried monolayers of parasitized erythrocytes (12).

The stage specificity of FIRA is therefore in some ways analogous to RESA (2). Hybridization of cDNA prepared from mRNA of highly purified merozoites to the array of 133 colonies revealed another parallel with RESA. All members of the Ag231 family hybridized to merozoite cDNA. Remarkably, the only other clones in this array or in a separate array of 78 antigen positive clones, that hybridize to merozoite cDNA encode RESA (2,13). Hence FIRA and RESA mRNAs are unusual among mRNAs for P.falciparum antigens in that they are greatly enriched in merozoites.

FIRA Sequence

The chromosomal clone encompassing the AhaIII fragment, cloned in λgt10 and designated Ag231.5 has been fully sequenced. The gene contains an intervening sequence and is remarkably like RESA in overall structure. Exon 1 consists of a segment that may be a signal peptide (although it is very short), then a

region of hydrophilic amino acid followed by a stretch of 32 uncharged amino acids. The intervening sequence is located immediately adjacent to this relatively hydrophobic segment. The remaining sequence is composed of blocks of repetitive and interspersed non-repetitive sequences. In all cases, the repetitive sequences occur as groups of 13 hexamers, but the most 5' group of these lack interspersed non-repetitive sequences - i.e. there is a block of 39 hexamers. It appears that a deletion at the 3' end has altered the linker - Aha join, so the structure at the 3' end is uncertain.

Cross-reactions amongst repeats

Human antibodies affinity-purified on Ag231.6 (FIRA) when tested in an ELISA gave a very strong signal on Ag231.6, a weaker but very definite signal on Ag13.1.7.5 (RESA 5' repeat), and no signal on Ag13 (RESA 3' repeat) (Figure 10). This cross-reaction is consistent with the sequence homology between the repeats in these otherwise distinct antigens.

A full description of the preparation of recombinant DNA molecules, and of recombinant DNA cloning vehicles and vectors, of host cell-cloning vehicle combinations, and of the expression of polypeptides by host cells is contained in International Patent Specification No. PCT/AU84/00016. This specification also describes in detail the use of DNA molecules and polypeptides expressed thereby in serological diagnosis, and in the preparation of single and multivalent vaccines for stimulating protective antibodies against Plasmodia. That description is equally applicable to the present invention and is incorporated herein by reference.

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CLAIMS:

1. A DNA molecule comprising a nucleotide sequence substantially corresponding to all or a portion of the base sequence coding for an antigen of P.falciparum selected from the group consisting of the Ring-Infected Erythrocyte Surface Antigen (RESA), the Falciparum Interspersed Repeat Antigen (FIRA), and other antigens of P.falciparum cross-reactive therewith.

2. A DNA molecule according to claim 1, wherein said nucleotide sequence codes for a polypeptide of P.falciparum which substantially corresponds to the RESA antigen of P.falciparum.

3. A DNA molecule according to claim 1, wherein said nucleotide sequence codes for a polypeptide of P.falciparum which substantially corresponds to the FIRA antigen of P.falciparum.

4. A DNA molecule according to claim 1, wherein said nucleotide sequence is characterised by at least a portion thereof comprising a base sequence substantially as shown in Figure 1.

5. A DNA molecule according to claim 1, wherein said nucleotide sequence is characterised by at least a portion thereof comprising a base sequence substantially as shown in Figure 7.

6. A DNA molecule comprising a nucleotide sequence capable of being expressed as at least one polypeptide displaying the antigenicity of an antigen of P.falciparum selected from the group consisting of the

RESA antigen, the FIRA antigen, and other antigens of P.falciparum cross-reactive therewith.

7. A recombinant DNA molecule comprising a nucleotide sequence according to any one of claims 1 to 6, operatively linked to an expression control sequence.

8. A recombinant DNA cloning vehicle or vector capable of expressing all or a portion of at least one polypeptide or protein of P.falciparum, and having inserted therein a nucleotide sequence according to any one of claims 1 to 6, said sequence being operatively linked to an expression control sequence.

9. A recombinant DNA cloning vehicle or vector according to claim 8, characterised in that said nucleotide sequence and said expression control sequence are inserted into a bacteriophage.

10. A recombinant DNA cloning vehicle or vector according to claim 9, characterised in that said bacteriophage is bacteriophage λAmp 3.

11. A host cell containing a recombinant DNA molecule according to claim 7, or a recombinant DNA cloning vehicle or vector according to claim 8.

12. A synthetic peptide or polypeptide displaying the antigenicity of all or a portion of an antigen of P.falciparum selected from the group consisting of the RESA antigen, the FIRA antigen, and other antigens of P.falciparum cross-reactive therewith.

13. A synthetic peptide or polypeptide according to claim 12, characterised in that it displays the antigenicity of all or a portion of the RESA antigen of P.falciparum.

14. A synthetic peptide or polypeptide according to claim 12, characterised in that it displays the antigenicity of all or a portion of the FIRA antigen of P.falciparum.

15. A fused polypeptide comprising a polypeptide sequence displaying the antigenicity of an antigen of P.falciparum selected from the group consisting of the RESA antigen, the FIRA antigen, and other antigens of P.falciparum cross-reactive therewith as the C-terminal sequence, and an additional polypeptide as the N-terminal sequence fused thereto.

16. A fused polypeptide according to claim 15, wherein the additional polypeptide is a polypeptide coded for by the DNA of a recombinant DNA cloning vehicle or vector.

17. A composition for stimulating immune responses against P.falciparum antigens in a mammal, comprising at least one polypeptide displaying the antigenicity of an antigen of P.falciparum selected from the group consisting of the RESA antigen, the FIRA antigen, and other antigens of P.falciparum cross-reactive therewith, together with a pharmaceutically acceptable carrier therefor.

18. A composition according to claim 17, further comprising an adjuvant.

19. A composition for stimulating immune responses against P.falciparum antigens in a mammal, comprising a virus or microorganism in association with a pharmaceutically acceptable carrier, the virus or microorganism having inserted therein a DNA molecule comprising a nucleotide sequence capable of being expressed at least one polypeptide displaying the antigenicity of an antigen of P.falciparum selected from the group consisting of the RESA antigen, the FIRA antigen, and other antigens of P.falciparum cross-reactive therewith.

20. A method of stimulating immune responses against P.falciparum antigens in a mammal, which comprises administering a composition according to claim 17 or claim 18 to said mammal.

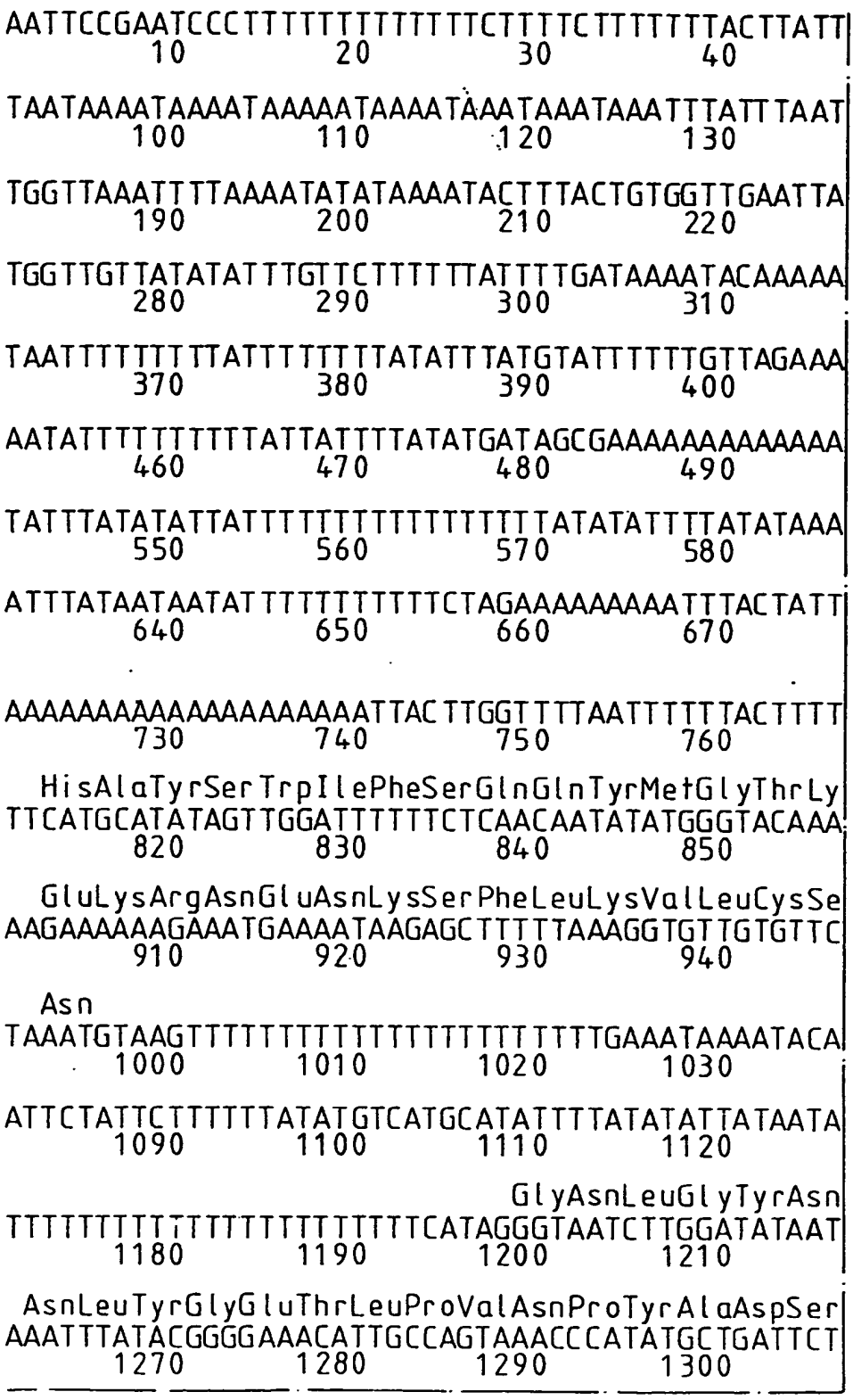
AATTCCGAATCCCTTTTTTTTTTTTTCTTTTCTTTTTTTACTTATT
10 20 30 40
TAATAAAATAAAATAAAAATAAAATAAATAAATAAATTTATTTAAT
100 110 120 130
TGGTTAAATTTTAAAATATATAAAATACTTTACTGTGGTTGAATTA
190 200 210 220
TGGTTGTTATATATTTGTTCTTTTTTATTTTGATAAAATACAAAAA
280 290 300 310
TAATTTTTTTTTATTTTTTTTTATATTTATGTATTTTTTGTTAGAAA
370 380 390 400
AATATTTTTTTTTTATTATTTTATATGATAGCGAAAAAAAAAAAAA
460 470 480 490
TATTTATATATTATTTTTTTTTTTTTTTTTATATATTTTATATAAA
550 560 570 580
ATTTATAATAATATTTTTTTTTTTTCTAGAAAAAAAAATTTACTATT
640 650 660 670
AAAAAAAAAAAAAAAAAAAATTACTTGGTTTTAATTTTTTACTTTT
730 740 750 760
HisAlaTyrSerTrpIlePheSerGlnGlnTyrMetGlyThrLy
TTCATGCATATAGTTGGATTTTTTCTCAACAATATATGGGTACAAA
820 830 840 850
GluLysArgAsnGluAsnLysSerPheLeuLysValLeuCysSe
AAGAAAAAAGAAATGAAAATAAGAGCTTTTTAAAGGTGTTGTGTTC
910 920 930 940
Asn
TAAATGTAAGTTTTTTTTTTTTTTTTTTTTTTTGAAATAAAATACA
1000 1010 1020 1030
ATTCTATTCTTTTTTATATGTCATGCATATTTTATATATTATAATA
1090 1100 1110 1120
GlyAsnLeuGlyTyrAsn
TTTTTTTTTTTTTTTTTTTTTTTTCATAGGGTAATCTTGGATATAAT
1180 1190 1200 1210
AsnLeuTyrGlyGluThrLeuProValAsnProTyrAlaAspSer
AAATTTATACGGGGAAACATTGCCAGTAAACCCATATGCTGATTCT
1270 1280 1290 1300

FIG. 1(A). a.

```
ATATTTTTTTATTAAGTGAAAAAAAAAAAAAAAAAAAATAATAA
  50         60         70         80         90

AATTTTTATATAGATTTAATATATATCGGTTGATAGATTTCGTT
 140        150        160        170        180

TTAAAAAAAAAAAATAATAAATAAATTAAAAGCTTCCTTATTCT
 230        240        250        260        270

AAAAATAATAAAACCTAATTATAAAAAAAAAATAAAAGTTCATA
 320        330        340        350        360

AAAAAAAAAAAAAAAAAAAGAAATAATTTATTTATAATATATA
 410        420        430        440        450

AAAAAAATAAAATAATTTTTAAAAATATTTTATTATTATTAAAA
 500        510        520        530        540

TAACATTTTCTATAAATTAAATATATTTTAATATATATATATAT
 590        600        610        620        630

TTTATATTTATATATATTATAATATTATTTAGACATATTATTAA
 680        690        700        710        720

                                        MetArgProPhe
ACATAATTTATAATAAGAAAATATCTAAATAATTATGAGACCTT
 770        780        790        800        810

sAsnValLysGluLysAsnProThrIleTyrSerPheAspAspGlu
AAATGTTAAGGAAAAAAATCCCACCATATATTCATTTGATGATG
 860        870        880        890        900

rLysArgGlyValLeuProIleIleGlyIleLeuTyrIleIleLeu
TAAACGTGGTGTTCTTCCAATTATTGGAATACTATATATCATTT
 950        960        970        980        990


TATTTTTTATATTTAATTTTTTATGTTAATGCTTATTTTATTTT
1040       1050       1060       1070       1080

CCGTTTTTAATAATATATAATATATCTTTGTTATTATATATAAT
1130       1140       1150       1160       1170

GlySerSerSerSerGlyValGlnPheThrAspArgCysSerArg
GGAAGTTCATCTTCTGGCGTACAATTTACTGATAGATGTTCAAG
1220       1230       1240       1250       1260

GluAsnProIleValValSerGlnValPheGlyLeuProPheGlu
GAAAACCCAATAGTTGTAAGTCAGGTATTTGGTTTACCCTTCGA
1310       1320       1330       1340       1350
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FIG. 1(A)b

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 LysProThrPheThrLeuGluSerProProAspIleAspHisThr
AAAACCTACGTTTACCTTAGAAAGTCCTCCTGATATTGATCATACA
      1360       1370       1380       1390

 TyrArgTyrSerAsnAsnTyrGluAlaIleProHisIleSerGlu
ATATCGATATTCTAATAACTATGAAGCCATTCCTCATATAAGTGAG
      1450       1460       1470       1480

 LysValAspAsnLeuGlyArgSerGlyGlyAspIleIleLysLys
AAAGGTTGATAACTTAGGAAGAAGTGGAGGAGACATTATAAAAAAA
      1540       1550       1560       1570

 TyrAspSerLeuLysGluLysLeuGlnLysThrTyrSerGlnTyr
ATATGATTCTTTAAAAGAAAAATTACAGAAAACTTACAGTCAGTAC
      1630       1640       1650       1660

 ThrGlnCysIleLysLeuIleAspGlnGlyGlyGluAsnLeuGlu
GACACAATGCATAAAACTTATTGATCAAGGAGGAGAGAACCTTGAA
      1720       1730       1740       1750

 LeuAsnLeuGluGluTyrArgArgLeuThrValLeuAsnGlnIle
TTTAAATCTTGAAGAATATAGAAGATTGACTGTGTTGAACCAAATC
      1810       1820       1830       1840

 IleMetAsnSerAspIleSerSerPheLysHisIleAsnGluLeu
AATTATGAATAGTGACATTTCTTCCTTTAAACATATAAATGAATTG
      1900       1910       1920       1930

 LysLysArgAlaGlnLysProLysLysLysLysSerArgArgGly
GAAGAAAAGAGCTCAAAAACCGAAGAAGAAAAAAAGTAGAAGAGGA
      1990       2000       2010       2020

 GlnGluGluProValGlnThrValGlnGluGlnGlnValAsnGlu
ACAAGAAGAACCAGTCCAAACCGTTCAAGAACAACAAGTAAATGAA
      2080       2090       2100       2110

 AlaIleAsnTyrTyrAspThrValLysAspGlyValThrLeuAsp
AGCTATTAATTATTATGATACCGTAAAAGATGGTGTATACTTAGAC
      2170       2180       2190       2200

 AspLeuGluLysGlnLysTyrMetAspMetLeuAspThrSerGlu
TGATTTGGAAAAACAAAAATATATGGATATGTTAGATACATCTGAA
      2260       2270       2280       2290

 GluHisValGluGluHisThrAlaAspAspGluHisValGluGlu
TGAACATGTAGAAGAACACACTGCTGATGACGAACATGTAGAAGAA
      2350       2360       2370       2380

 AspGluHisValGluGluProThrValAlaGluGluHisValGlu
TGATGAACACGTAGAAGAACCAACTGTTGCTGAAGAACATGTAGAA
      2440       2450       2460       2470
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FIG. 1(B)a.

SUBSTITUTE SHEET

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AsnIleLeuGlyPheAsnGluLysPheMetThrAspValAsnArg
AATATTTTGGGTTTTAATGAGAAGTTCATGACTGATGTAAATAG
1400       1410       1420       1430       1440

PheAsnProLeuIleValAspLysValLeuPheAspTyrAsnGlu
TTCAATCCACTTATTGTAGATAAAGTTCTTTTCGACTATAACGA
1490       1500       1510       1520       1530

MetGlnThrLeuTrpAspGluIleMetAspIleAsnLysArgLys
ATGCAAACTTTATGGGATGAAATAATGGATATTAATAAAAGAAA
1580       1590       1600       1610       1620

LysValGlnTyrAspMetProLysGluAlaTyrGluSerLysTrp
AAGGTTCAATATGATATGCCAAAAGAAGCATATGAGAGCAAATG
1670       1680       1690       1700       1710

GluArgLeuAsnSerGlnPheLysAsnTrpTyrArgGlnLysTyr
GAAAGATTGAACTCACAATTTAAAAACTGGTACAGGCAGAAATA
1760       1770       1780       1790       1800

AlaTrpLysAlaLeuSerAsnGlnIleGlnTyrSerCysArgLys
GCTTGGAAAGCTTTATCCAACCAAATTCAATATTCATGCAGAAA
1850       1860       1870       1880       1890

LysSerLeuGluHisArgAlaAlaLysAlaAlaGluAlaGluMet
AAAAGTTTAGAACACAGAGCCGCAAAAGCTGCAGAAGCAGAAAT
1940       1950       1960       1970       1980

TrpLeuCysCysGlyGlyGlyAspIleGluThrValGluProGln
TGGTTATGTTGTGGGGGGGGAGATATCGAAACAGTTGAACCACA
2030       2040       2050       2060       2070

TyrGlyAspIleLeuProSerLeuArgAlaSerIleThrAsnSer
TATGGTGATATATTACCATCATTAAGGGCCAGTATTACTAATTC
2120       2130       2140       2150       2160

HisGluThrSerAspAlaLeuTyrThrAspGluAspLeuLeuPhe
CATGAAACATCAGATGCTCTTTATACAGATGAAGATTTGTTATT
2210       2220       2230       2240       2250

GluGluSerValGluGluAsnGluGluGluHisThrValAspAsp
GAAGAATCTGTTGAAGAAAATGAAGAAGAACACACTGTTGATGA
2300       2310       2320       2330       2340

ProThrValAlaAspAspGluHisValGluGluProThrValAla
CCAACTGTTGCTGATGATGAACATGTAGAAGAACCAACTGTTGC
2390       2400       2410       2420       2430

GluProThrValAlaGluGluHisValGluGluProAlaSerAsp
GAACCAACTGTTGCTGAAGAACACGTAGAAGAACCAGCTAGTGA
2480       2490       2500       2510       2520
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FIG. 1(B)b.

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ValGlnGlnThrSerGluAlaAlaProThrIleGluIleProAsp
TGTTCAACAAACTTCAGAAGCAGCTCCAACAATTGAAATCCCCGAT
      2530       2540       2550       2560

AsnGluIleThrGluArgTyrPheLysLeuAlaGluAsnTyrTyr
GAACGAAATTACTGAACGTTATTTTAAGTTAGCTGAAAATTACTAT
      2620       2630       2640       2650

ValAsnGluAlaTyrGlnValLeuGlyAspIleAspLysLysArg
AGTCAACGAAGCCTACCAAGTTTTAGGAGATATTGATAAAAAAAGA
      2710       2720       2730       2740

MetAsnProSerIlePheTyrLeuLeuSerSerLeuGluLysPhe
TATGAATCCATCCATCTTTTATTTATTATCTAGTTTAGAAAAATTT
      2800       2810       2820       2830

PhePheGluLysArgLeuSerMetAsnAspLeuGluAsnLysSer
CTTTTTTGAAAAGAGATTATCTATGAATGATTTAGAGAATAAAAGT
      2890       2900       2910       2920

AlaHisValSerGluTyrLeuLeuAsnIleLeuGlnProCysIle
AGCACATGTATCTGAATATTTATTAAATATATTACAACCATGTATA
      2980       2990       3000       3010

GlyLeuLysGlySerArgPheAspIleProIleLeuGluSerLeu
AGGTTTAAAAGGATCTCGCTTTGATATACCAATATTAGAATCTTTA
      3070       3080       3090       3100

SerLysSerAlaLysLysLeuGlnGlnArgThrGlnAlaAsnLys
CTCAAAATCAGCTAAGAAACTTCAACAGAGAACCCAGGCTAATAAA
      3160       3170       3180       3190

GluTyrLeuGlySerSerGluGlnMetAsnSerIleThrTyrAsn
AGAATATTTAGGAAGTAGTGAACAAATGAATTCAATAACATACAAT
      3250       3260       3270       3280

AsnIleSerAspLeuSerTyrThrAspGlnLysGluIleLeuGlu
AAATATTTCAGATTTAAGTTATACAGATCAGAAGGAAATATTAGAA
      3340       3350       3360       3370

AsnThrAlaLeuAsnAlaAlaGluGlnLeuLeuSerAspAsnSer
GAACACAGCTTTAAATGCCGCTGAACAATTGTTGTCAGATAATTCA
      3430       3440       3450       3460

LeuSerSerIleMetGluArgTyrAlaGlyGlyLysArgAsnAsp
-ATTATCATCCATTATGGAGAGATATGCAGGTGGTAAAAGAAACGAT
      3520       3530       3540       3550
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FIG. 1(C).a.

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ThrLeuTyrTyrAspIleLeuGlyValGlyValAsnAlaAspMet
ACATTATATTACGATATATTAGGTGTTGGTGTTAATGCTGATAT
2570       2580       2590       2600       2610

ProTyrGlnArgSerGlySerThrValPheHisAsnPheArgLys
CCATACCAAAGATCAGGTTCTACTGTTTTCCACAACTTTAGGAA
2660       2670       2680       2690       2700

TrpTyrAsnLysTyrGlyTyrAspGlyIleLysGlnValAsnPhe
TGGTACAATAAATACGGATATGATGGAATAAAACAAGTCAACTT
2750       2760       2770       2780       2790

LysAspPheThrGlyThrProGlnIleValThrLeuLeuArgPhe
AAAGATTTTACCGGAACACCCCAAATAGTAACTCTTTTGAGATT
2840       2850       2860       2870       2880

GluHisLeuLeuLysPheMetGluGlnTyrGlnLysGluArgGlu
GAACATTTATTAAAATTTATGGAACAATATCAAAAAGAAAGAGA
2930       2940       2950       2960       2970

AlaGlyAspSerLysTrpAsnValProIleIleThrLysLeuGlu
GCTGGTGATTCAAAATGGAATGTACCAATTATAACAAAACTTGA
3020       3030       3040       3050       3060

ArgTrpIlePheLysHisValAlaLysThrHisLeuLysLysSer
AGATGGATATTCAAACATGTCGCTAAAACACATTTGAAAAAATC
3110       3120       3130       3140       3150

GlnGluLeuAlaAsnIleAsnAsnAsnLeuMetSerThrLeuLys
CAAGAATTAGCAAATATAAATAATAACCTAATGAGTACATTGAA
3200       3210       3220       3230       3240

PheGluAsnIleAsnSerAsnValAspAsnGlyAsnGlnSerLys
TTCGAAAACATCAATTCCAATGTTGATAACGGAAACCAATCAAA
3290       3300       3310       3320       3330

LysIleValSerTyrIleValAspIleSerLeuTyrAspIleGlu
AAAATTGTTAGTTATATAGTAGATATTTCCCTTTATGATATAGA
3380       3390       3400       3410       3420

ValAspGluLysThrLeuLysLysArgAlaGlnSerLeuLysLys
GTAGATGAAAAAACTCTTAAAAAGAGAGCTCAATCATTAAAAAA
3470       3480       3490       3500       3510

LysLysSerLysAsnPheAspThrLysAspIleValGlyTyrIle
AAAAAATCAAAAAATTTTGATACCAAAGATATTGTAGGATATAT
3560       3570       3580       3590       3600
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FIG. 1(C).b.

```
 MetHisGlyIleSerThrIleAsnThrGluMetLysAsnGlnAsn
TATGCATGGAATTAGCACAATTAATACAGAAATGAAAAACCAAAAT
       3610       3620       3630       3640

 GluHisAspAlaGluGluAsnValGluHisAspAlaGluGluAsn
AGAACATGATGCTGAAGAAAATGTAGAACATGATGCTGAAGAAAAT
       3700       3710       3720       3730

 AsnValGluHisAspAlaGluGluAsnValGluGluAsnValGlu
AAATGTAGAACATGATGCTGAAGAAAATGTAGAAGAAAATGTTGAA
       3790       3800       3810       3820

 GluAsnValGluGluValGluGluAsnValGluGluAsnValGlu
AGAAAATGTTGAAGAAGTAGAAGAAAATGTAGAAGAAAATGTAGAA
       3880       3890       3900       3910

 GluGluAsnValGluGluAsnValGluGluAsnValGluGluTyr
TGAAGAAAATGTAGAAGAAAATGTAGAAGAAAATGTTGAAGAATAT
       3970       3980       3990       4000

 ValGluGluAsnValGluGluAsnValGluGluAsnValGluGlu
TGTAGAAGAAAATGTTGAAGAAAATGTAGAAGAAAATGTTGAAGAA
       4060       4070       4080       4090

 AsnValGluGluAsnValGluGluAsnValGluGluTyrAspGlu
GAATGTTGAAGAGAATGTTGAAGAGAATGTTGAAGAATATGATGAA
       4150       4160       4170       4180

AATATATATATTAAAGTTTTAATTTTTATAAACAGAATAATACTAA
       4240       4250       4260       4270

TATGAAAAAGAAATGTGTGTTTTTTTTCTTTTTTTTTTTTTTTTT
       4330       4340       4350       4360

ATTTATTTCTTTTAATTTGCGATATGATATTACATGTAAATAATAA
       4420       4430       4440       4450

CATTGTAATTTATATTGTTGTATTTGTTTTAATGTTTTCACATTTT
       4510       4520       4530       4540

C
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FIG. 1(D).a.

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GluAsnValProGluHisValGlnHisAsnAlaGluGluAsnVal
GAAAATGTACCAGAACATGTACAACATAATGCTGAAGAAAATGT
3650       3660       3670       3680       3690

ValGluHisAspAlaGluGluAsnValGluHisAspAlaGluGlu
GTAGAACATGATGCTGAAGAAAATGTAGAACATGATGCTGAAGA
3740       3750       3760       3770       3780

GluValGluGluAsnValGluGluAsnValGluGluAsnValGlu
GAAGTAGAAGAAAATGTAGAAGAAAATGTAGAAGAAAATGTAGA
3830       3840       3850       3860       3870

GluAsnValGluGluAsnValGluGluAsnValGluGluAsnVal
GAAAATGTAGAAGAAAATGTTGAAGAAAATGTTGAAGAAAATGT
3920       3930       3940       3950       3960

AspGluGluAsnValGluGluValGluGluAsnValGluGluAsn
GATGAAGAAAATGTTGAAGAAGTAGAAGAAAATGTAGAAGAAAA
4010       4020       4030       4040       4050

ValGluGluAsnValGluGluAsnValGluGluAsnValGluGlu
GTAGAAGAAAATGTAGAAGAAAATGTAGAAGAAAATGTAGAAGA
4100       4110       4120       4130       4140

GluAsnValGluGluHisAsnGluGluTyrAspGlu
GAAAATGTTGAAGAACACAATGAAGAATATGATGAATAAAAAAA
4190       4200       4210       4220       4230

ATGAACGATTTCTCTTTATGAAAATAAAATATTTAAAACAGATA
4280       4290       4300       4310       4320

TTTTCTTGCATGAATGTATTTGTTATTTTAAAATTTGTTCTTAT
4370       4380       4390       4400       4410

TTTGTAATTTATATTTTTTCTTTTCTTTTTATTTTTATTTTATT
4460       4470       4480       4490       4500

ATTTGTCTTTTTTTTATTATAATTAAAAAAAAAAAAACGGAATT
4550       4560       4570       4580       4590
```

FIG. 1(D).b.

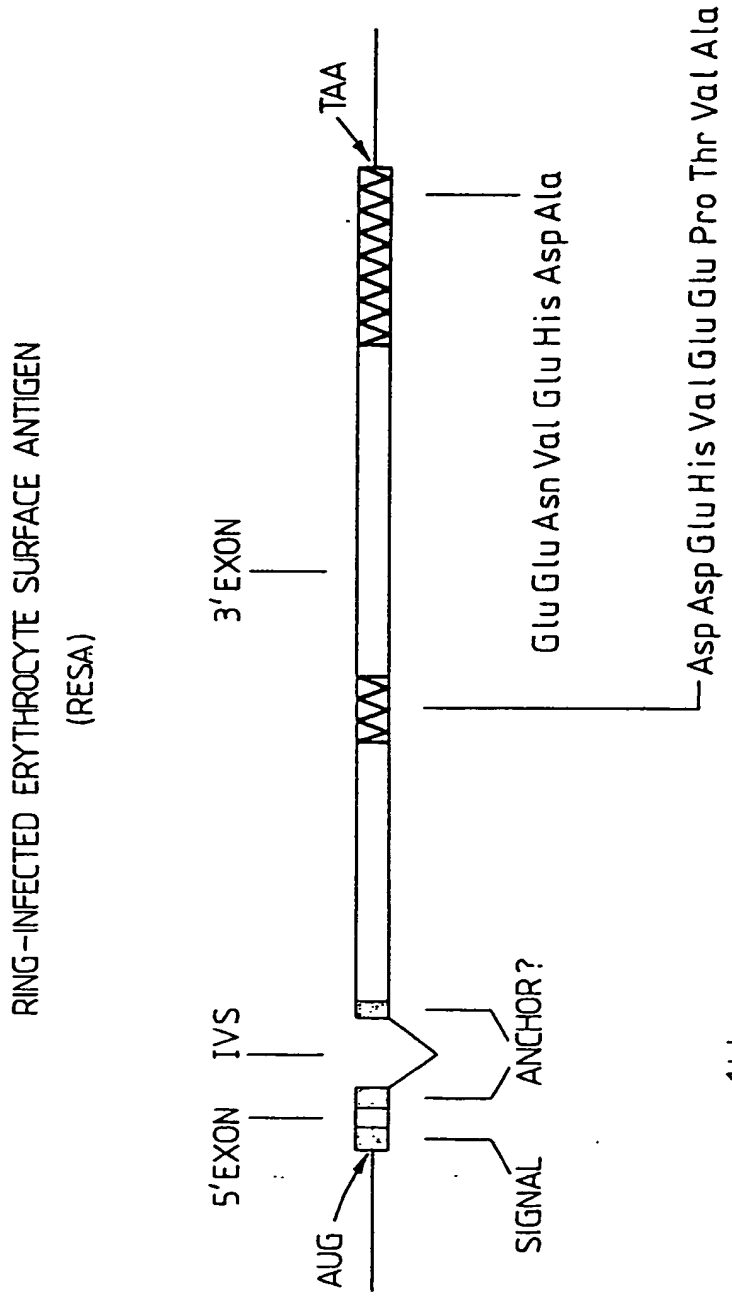

RING-INFECTED ERYTHROCYTE SURFACE ANTIGEN
(RESA)
5'EXON
IVS
3'EXON
AUG
SIGNAL
ANCHOR?
TAA
Asp Asp Glu His Val Glu Glu Pro Thr Val Ala
Glu Glu Asn Val Glu His Asp Ala
1kb

FIG. 2.

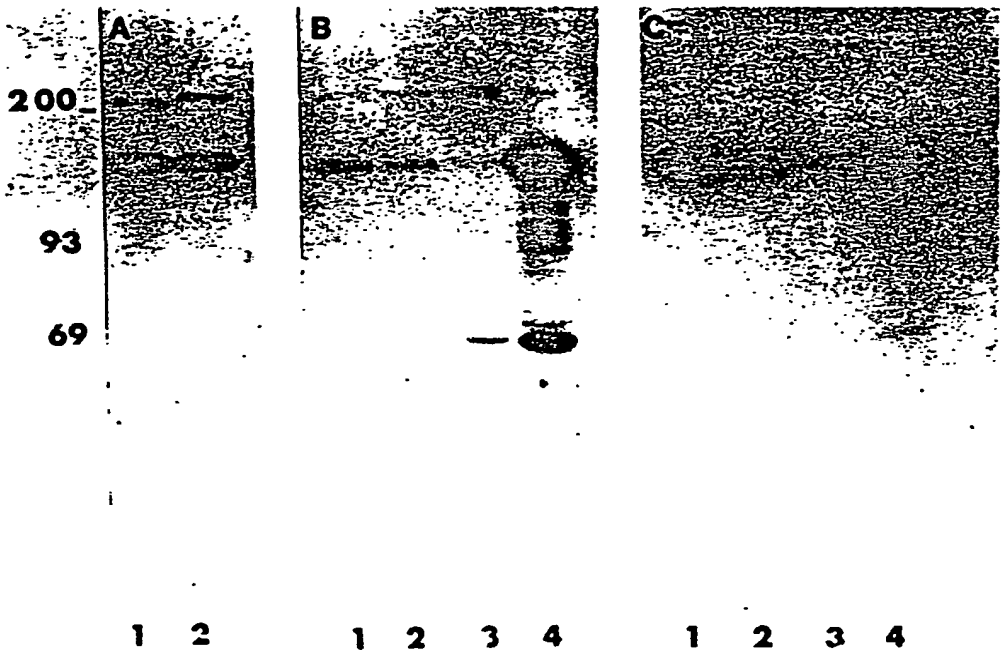


FIG. 3.

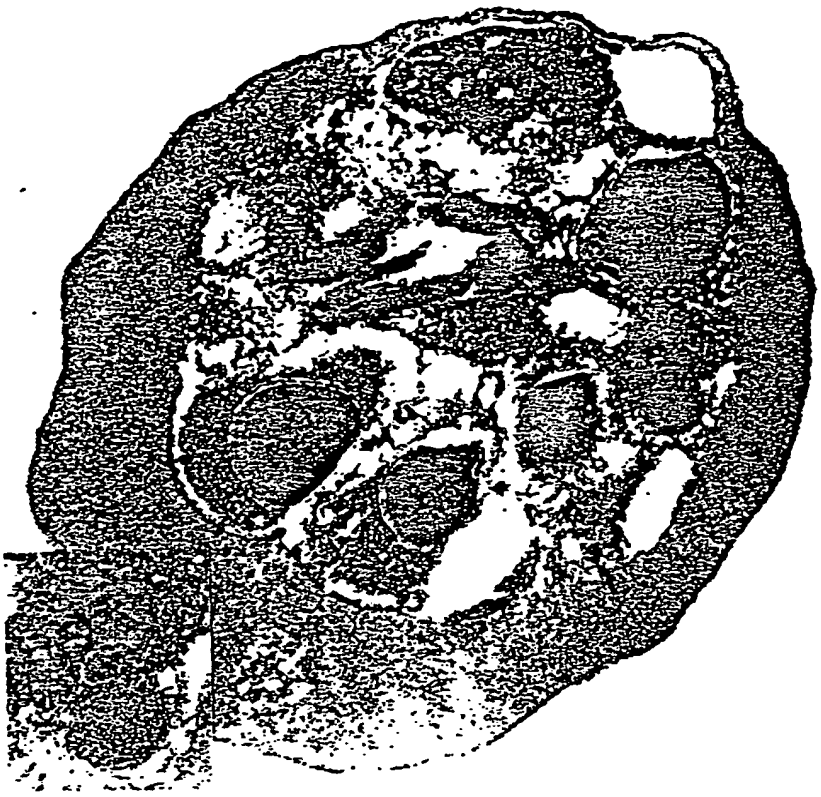


FIG. 4.

FIG. 5.

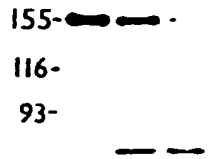

155-
116-
93-
1 2 3

FIG. 6.

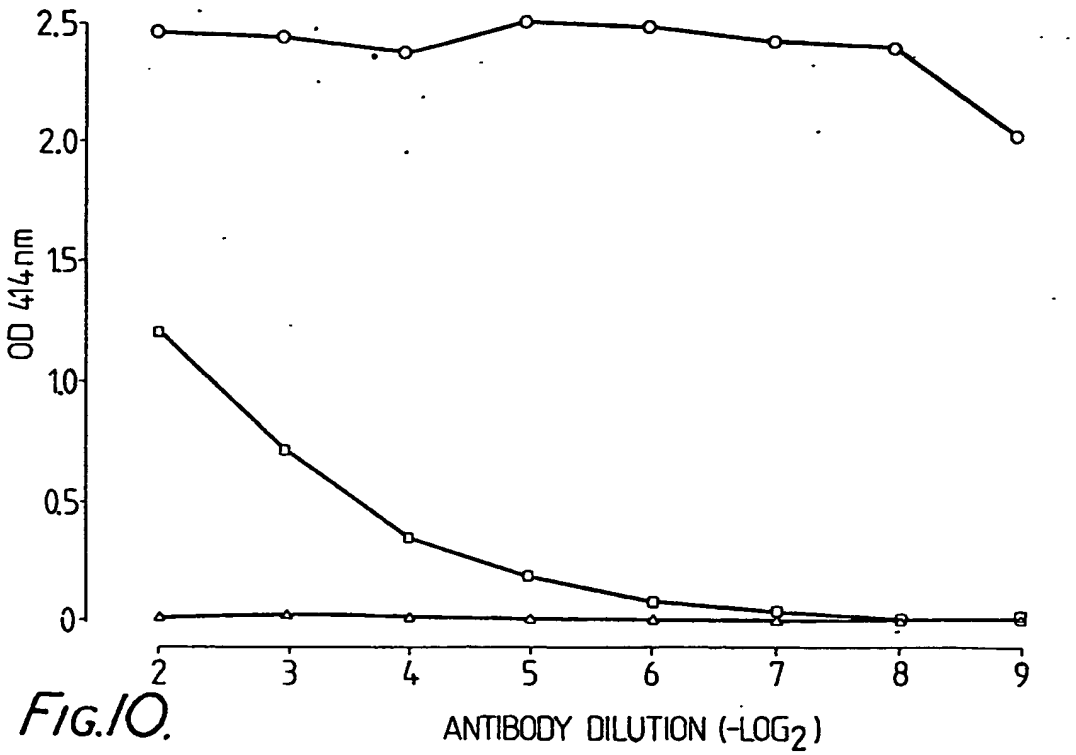

2.5
2.0
1.5
1.0
0.5
0
OD 414nm
2
3
4
5
6
7
8
9
ANTIBODY DILUTION (-LOG2)

FIG. 10.

```
AAATATAAGTGTATATAAAAAAAAATATAATCATATTTTTTTTATT
        10        20        30        40

  GlnAsnLysAlaSerSerProSerIleAsnValAspGluTyrSe
CACAAAATAAAGCTTCTAGTCCAAGCATAAATGTAGATGAATATTC
       100       110       120       130

  ThrAsnLeuThrProAspGlnIleSerAlaLeuAsnAlaHisLe
TTACGAATCTAACACCTGATCAAATAAGTGCATTGAATGCGCATTT
       190       200       210       220

  AsnAsnGluAsnGluValAsnProLeuValProSerSerIleSe
CAAATAACGAAAATGAAGTAAATCCATTAGTACCATCATCAATTTC
       280       290       300       310

  IleSerIleValAsnPheCys
TTATTTCTATTGTTAATTTTTGTGTAAGGAAAATAAAATAAAATAA
       370       380       390       400

AATATTCATGTATAAAATAATTTTAACCTATCATACATGTTTTAAT
       460       470       480       490

                     ArgLysLysSerGlnThrTyrAsnLys
TTTTATATTTTTTCTTTAGCGAAAGAAATCACAAACATACAATAAA
       550       560       570       580

 AlaThrGlnGlnGluAsnSerAsnGlnAsnLysGluIleAsnGlu
TGCAACACAGCAAGAAAATAGTAATCAAAATAAGGAAATTAATGAA
       640       650       660       670

 ThrValThrThrGlnAlaAlaAlaThrProGlnGluThrValGlu
AACAGTCACAACACAAGCAGCAGCCACACCACAAGAAACAGTCGAA
       730       740       750       760

 ProValThrThrGlnGluProIleThrValGlnGluProValThr
ACCTGTAACAACACAAGAACCTATAACGGTACAAGAACCAGTCACA
       820       830       840       850

 ProValThrValGlnGluProValThrValGlnGluProValThr
ACCAGTCACAGTACAAGAACCAGTCACAGTACAAGAACCAGTCACA
       910       920       930       940
```

FIG. 7(A).a.

```
                                                        MetGluSer
TTGTTCTTGATACCTTACAATAGTATATAATATAGAAATGGAAT
  50         60         70         80         90

rSerLeuThrSerAsnAsnGluAsnProGlnAsnThrAlaThrLeu
AAGTCTTACAAGCAACAATGAAAATCCACAAAATACCGCTACTC
 140        150        160        170        180

uProAsnGluIleAsnIleGluThrIleThrSerThrLeuThrThr
ACCAAATGAAATAAATATAGAAACAATTACTTCTACATTGACAA
 230        240        250        260        270

rAsnThrLeuAspThrLeuThrPheTyrGlnLeuIleLeuIleIle
AAATACCCTAGATACATTGACATTTTATCAATTAATTTTGATAA
 320        330        340        350        360


ATATTAATAATAATCATAATAATAATAAATGTTATATAATAAAA
 410        420        430        440        450

TATACATATTCATTATAATATTGTAAATATTTATATTCATATAT
 500        510        520        530        540

AsnPheGluGluLysPheAsnLeuAlaSerValGlnSerSerAsn
AATTTTGAAGAAAAATTTAATTTAGCAAGCGTTCAAAGTTCTAA
 590        600        610        620        630

ValLysGluSerSerGlnThrGlnProProValThrProGlnGlu
GTAAAAGAGTCTTCTCAAACACAACCACCAGTGACACCACAAGA
 680        690        700        710        720

ThrGlnGluProValThrIleGluGluProValThrThrGlnGlu
ACACAAGAACCAGTAACAATAGAAGAACCAGTAACAACACAAGA
 770        780        790        800        810

ValGlnGluProValThrValGlnGluProValThrValGlnGlu
GTACAAGAACCAGTCACAGTACAAGAACCAGTCACAGTACAAGA
 860        870        880        890        900

ValGlnGluProValThrSerGlnGluProValThrProGlnGlu
GTACAAGAACCTGTGACATCACAAGAACCTGTGACACCACAAGA
 950        960        970        980        990
```

FIG. 7(A).b.

```
 ProValThrProGlnGluProValThrProGlnGluProValThr
ACCTGTGACACCACAAGAACCTGTGACACCACAAGAACCTGTGACA
      1000       1010       1020       1030

 ProValThrIleGluGluProValThrThrGlnGluProValThr
ACCAGTAACAATAGAAGAACCAGTAACAACACAAGAACCAGTAACA
      1090       1100       1110       1120

 ProValThrThrGlnGluProValThrThrGlnGluProValThr
ACCAGTAACAACACAAGAACCAGTAACAACACAAGAACCAGTAACA
      1180       1190       1200       1210

 ProValThrValGluGluHisIleAspGluLysLysGlySerGlu
ACCAGTAACAGTAGAAGAACATATTGATGAGAAAAAAGGATCAGAA
      1270       1280       1290       1300

 LysSerHisThrLysLysLysLysSerSerTrpLeuLysPheGly
AAAATCTCACACAAAAAAAAAAAAAAGCAGCTGGCTTAAATTTGGA
      1360       1370       1380       1390

 SerLeuGluSerValLysGlnAsnAlaAspGluGlnLysGluGln
TTCATTAGAAAGTGTAAAACAAAATGCTGATGAACAAAAAGAACAA
      1450       1460       1470       1480

 IleGlnGluProThrAlaThrGlnGluProProThrThrGlnGlu
AATACAAGAACCAACCGCAACACAAGAACCACCCACAACACAAGAA
      1540       1550       1560       1570

 GluGlnGluProThrThrThrGlnGluThrValThrAlaGlnGlu
AGAACAAGAACCAACAACAACACAAGAAACAGTAACAGCACAAGAA
      1630       1640       1650       1660

 ThrGlnGluLeuIleAlaThrGlnGluProSerThrThrGlnGlu
AACACAAGAACTAATCGCAACACAAGAACCATCCACAACACAAGAA
      1720       1730       1740       1750

 SerArgLeuSerGluGluThrGluGluLysSerHisThrLysLys
AAGCAGATTATCGGAAGAAACTGAAGAAAAATCTCACACAAAAAAA
      1810       1820       1830       1840
```

FIG. 7(B).a.

```
ProGlnGluProValThrThrGlnGluProValThrThrGlnGlu
CCACAAGAACCAGTAACAACACAAGAACCAGTAACAACACAAGA
1040       1050       1060       1070       1080

IleGluGluProValThrThrGlnGluProValThrIleGluGlu
ATAGAAGAACCAGTAACAACACAAGAACCAGTAACAATAGAAGA
1130       1140       1150       1160       1170

ThrGlnGluProValThrThrGlnGluProValThrThrGlnGlu
ACACAAGAACCAGTAACAACACAAGAACCAGTAACAACACAAGA
1220       1230       1240       1250       1260

GlyAspAsnIleSerLeuSerSerLeuSerGluGluThrGluGlu
GGTGATAACATTTCATTAAGCAGCTTATCGGAAGAAACTGAAGA
1310       1320       1330       1340       1350

ArgGlyAsnLysAsnAspLysLysSerLysAsnGluLysLysPro
AGAGGAAATAAAAATGACAAAAAAAGTAAAAACGAAAAAAAACC
1400       1410       1420       1430       1440

ProThrAspSerGlnIleSerValAsnAlaGlnAspSerValThr
CCTACAGATTCACAAATATCTGTTAATGCGCAAGATTCAGTAAC
1490       1500       1510       1520       1530

LeuThrAlaThrGlnGluProThrThrThrGlnGluThrValThr
CTAACCGCAACACAAGAACCAACCACGACACAAGAAACAGTAAC
1580       1590       1600       1610       1620

ProIleThrThrGlnGluProValThrAlaGlnGluProValThr
CCTATAACTACGCAAGAACCTGTTACAGCTCAAGAACCAGTCAC
1670       1680       1690       1700       1710

HisAlaAspGluLysLysAlaSerGluGlyAspAsnIleSerLeu
CATGCTGATGAGAAGAAAGCATCAGAAGGTGATAACATTTCATT
1760       1770       1780       1790       1800

LysLysSerSerTrpLeuLysPheGlyArgGlyAsnLysAsnAsp
AAAAAAAGCAGCTGGCTTAAATTTGGAAGAGGAAATAAAAATGA
1850       1860       1870       1880       1890
```

FIG. 7(B). b.

```
 LysLysSerLysAsnGluLysLysProSerLeuGluSerValLys
CAAAAAAAGTAAAAACGAAAAAAAACCTTCATTAGAAAGTGTAAAA
      1900       1910       1920       1930

 SerValAsnAlaGlnAspSerValThrIleGlnGluProThrAla
ATCTGTTAATGCGCAAGATTCAGTAACAATACAAGAACCAACCGCA
      1990       2000       2010       2020

 ProThrThrThrGlnGluThrValThrGluGlnGluProThrThr
ACCAACCACGACACAAGAAACAGTAACAGAACAAGAACCAACAACA
      2080       2090       2100       2110

 ProValThrAlaGlnGluProValThrThrGlnGluLeuIleAla
ACCTGTTACAGCTCAAGAACCAGTCACAACACAAGAACTAATCGCA
      2170       2180       2190       2200

 AlaSerGluGlyAspAsnIleSerLeuSerArgLeuSerGluGlu
AGCATCAGAAGGTGATAACATTTCATTAAGCAGATTATCGGAAGAA
      2260       2270       2280       2290

 LysPheGlyArgGlyAsnLysAsnAspLysLysSerLysAsnGlu
TAAATTTGGAAGAGGAAATAAAAATGACAAAAAAAAGTAAAAACGAA
      2350       2360       2370       2380

 LysGluGlnProThrAspSerGlnIleSerValAsnAlaGlnAsp
AAAAGAACAACCTACAGATTCACAAATATCTGTTAATGCGCAAGAT
      2440       2450       2460       2470

 ThrGlnGluLeuThrAlaThrGlnGluProThrThrThrGlnGlu
AACACAAGAACTAACCGCAACACAAGAACCAACCACGACACAAGAA
      2530       2540       2550       2560

 AlaGlnGluProIleThrThrGlnGluProValThrAlaGlnGlu
AGCACAAGAACCTATAACTACGCAAGAACCTGTTACAGCTCAAGAA
      2620       2630       2640       2650

 ThrGlnGluHisAlaAspGluLysLysAlaSerGluGlyAspAsn
AACACAAGAACATGCTGATGAGAAGAAAGCATCAGAAGGTGATAAC
      2710       2720       2730       2740
```

FIG. 7(C)a.

```
GlnAsnAlaAspGluGlnLysGluGlnProThrAspSerGlnIle
CAAAATGCTGATGAACAAAAAGAACAACCTACAGATTCACAAAT
1940       1950       1960       1970       1980

ThrGlnGluProProThrThrGlnGluLeuThrAlaThrGlnGlu
ACACAAGAACCACCCACAACACAAGAACTAACCGCAACACAAGA
2030       2040       2050       2060       2070

ThrGlnGluThrValThrAlaGlnGluProIleThrThrGlnGlu
ACACAAGAAACAGTAACAGCACAAGAACCTATAACTACGCAAGA
2120       2130       2140       2150       2160

ThrGlnGluProSerThrThrGlnGluHisAlaAspGluLysLys
ACACAAGAACCATCCACAACACAAGAACATGCTGATGAGAAGAA
2210       2220       2230       2240       2250

ThrGluGluLysSerHisThrLysLysLysLysSerSerTrpLeu
ACTGAAGAAAAATCTCACACAAAAAAAAAAAAAAGCAGCTGGCT
2300       2310       2320       2330       2340

LysLysProSerLeuGluSerValLysGlnAsnAlaAspGluGln
AAAAAACCTTCATTAGAAAGTGTAAAACAAAATGCTGATGAACA
2390       2400       2410       2420       2430

SerValThrIleGlnGluProThrAlaThrGlnGluProProThr
TCAGTAACAATACAAGAACCAACCGCAACACAAGAACCACCCAC
2480       2490       2500       2510       2520

ThrValThrGluGlnGluProThrThrThrGlnGluThrValThr
ACAGTAACAGAACAAGAACCAACAACAACACAAGAAACAGTAAC
2570       2580       2590       2600       2610

ProValThrThrGlnGluLeuIleAlaThrGlnGluProSerThr
CCAGTCACAACACAAGAACTAATCGCAACACAAGAACCATCCAC
2660       2670       2680       2690       2700

IleSerLeuSerArgLeuSerGluGluThrGluGluLysSerHis
ATTTCATTAAGCAGATTATCGGAAGAAACTGAAGAAAAATCTCA
2750       2760       2770       2780       2790
```

FIG. 7(C).b.

```
 ThrLysLysLysLysSerSerTrpLeuLysPheGlyArgGlyAsn
CACAAAAAAAAAAAAAAGCAGCTGGCTTAAATTTGGAAGAGGAAAT
      2800       2810       2820       2830

 SerValLysGlnAsnAlaAspGluGlnLysGluGlnProThrAsp
AAGTGTAAAACAAAATGCTGATGAACAAAAAGAACAGCCTACAGAT
      2890       2900       2910       2920

 ProIleThrAlaGlnGluThrValThrAspGlnGluProIleThr
ACCTATTACAGCTCAAGAAACTGTTACAGATCAAGAACCTATAACA
      2980       2990       3000       3010

 ThrValThrSerLeuValProAsnArgAsnThrArgAsnSerAsn
AACGGTTACTTCTCTTGTTCCGAATCGCAACACAAGAAACAGTAAC
      3070       3080       3090       3100

 ProValThrAlaGlnGluProValThrThrGlnGlu
ACCTGTTACAGCTCAAGAACCAGTGACAACACAAGAA
      3160       3170       3180
```

FIG. 7(D).a.

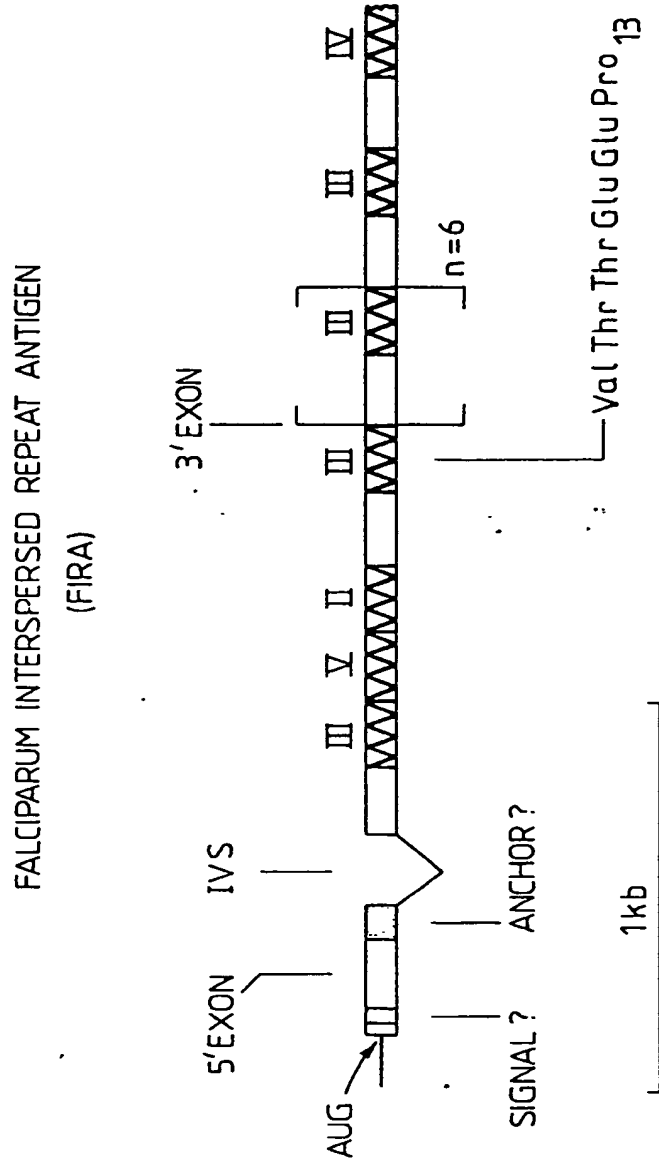
```
LysAsnAspLysLysSerLysAsnGluLysLysProSerLeuGlu
AAAAATGACAAAAAAAGTAAAAACGAAAAAAAACCTTCATTAGA
2840       2850       2860       2870       2880

SerGlnIleSerValAsnAlaGlnAspSerValThrThrGlnGlu
TCACAAATATCTGTTAATGCACAAGATTCAGTAACAACTCAAGA
2930       2940       2950       2960       2970

ThrGluGluProLeuThrThrGlnGluThrValThrThrGlnGlu
ACTGAAGAACCCTTAACCACACAAGAAACGGTTACAACACAAGA
3020       3030       3040       3050       3060

ArgThrArgThrIleThrThrGlnGluProIleThrThrGlnGlu
AGAACAAGAACTATAACGACACAGGAACCTATAACGACACAAGA
3110       3120       3120       3140       3150
```

FIG. 7(D).b.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(FIRA)
3'EXON
n=6
Val Thr Thr Glu Glu Pro 13
IV
III
III
II
V
III
IVS
5'EXON
AUG
SIGNAL ?
ANCHOR ?
1kb

FIG. 8.

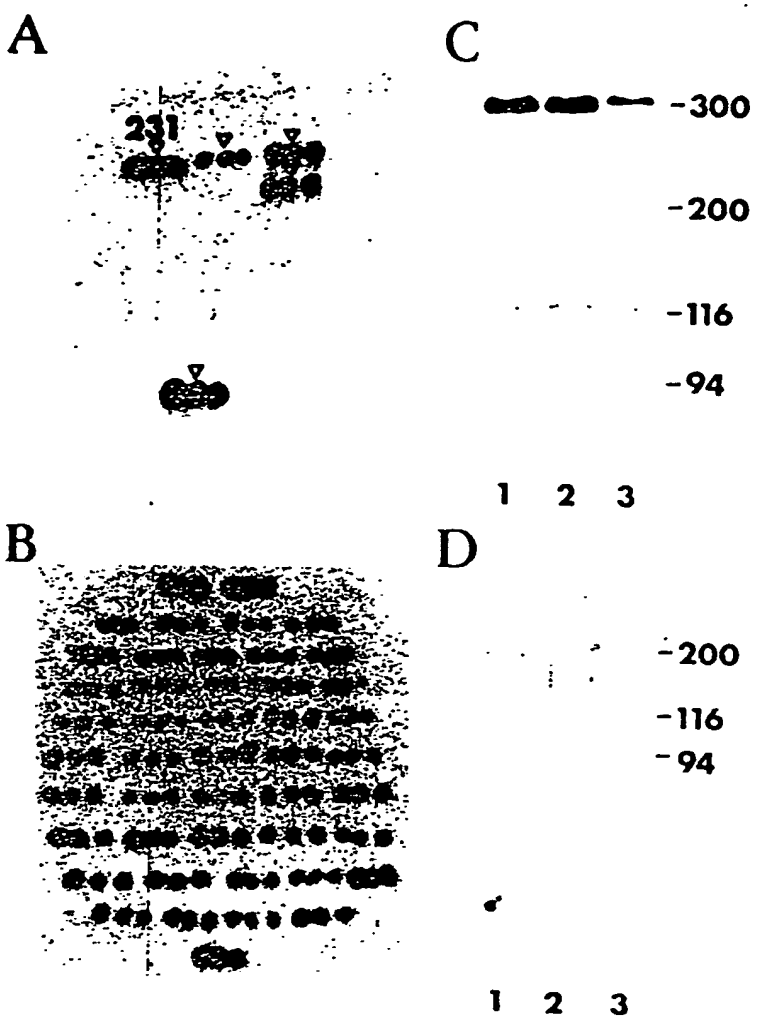

FIG. 9.

INTERNATIONAL SEARCH REPORT

International Application No PCT/AU 85/00223

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[4] C07H 21/04 // C12N 15/00, 1/20, C12P 19/34, 21/02, C07K 13/00, C07G 17/00, A61K 39/015, G01N 33/53 (C12N 1/20, C12R 1:19, 1:90)

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC	C07H 21/04
US Cl.	536/27, 536/28, 536/29

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

AU: IPC as above

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	AU,A, 23842/84 (THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH) 9 August 1984 (09.08.84) See especially claim 7; page 6 lines 20-25; and page 6 line 30 to page 7 line 2	(1-6)
X	WO,A1, 84/02917 (THE WALTER AND ELIZA HALL INSTITUTE OF MEDICAL RESEARCH) 2 August 1984 (02.08.84)	(1-6)
A,P	AU,A, 39046/85 (THE WELLCOME FOUNDATION LIMITED) 5 September 1985 (05.09.85)	(1-6)

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
11 December 1985 (11.12.85)	20 DECEMBER 1985 (20.12.85)
International Searching Authority	**Signature of Authorized Officer**
Australian Patent Office	R.M.F. BOYS

Form PCT/ISA/210 (second sheet) (January 1985)

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 85/00223

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members		
AU 39046/85	DK 799/85	EP 154454	GB 8404692
	GB 8504429	GB 2154592	IL 74409
	GB 8424340		
AU 23842/84	DK 4674/84	EP 134799	GB 2143830
	IL 70776	NO 843872	PT 78032
	WO 8402917	JP 60500478	FI 843797
	GB 8424104	ZA 840605	
WO 84/02917	DK 4674/84	EP 134799	FI 843797
	GB 8424104	GB 2143830	IL 70776
	JP 60500478	NO 843872	PT 78032
	ZA 840605		

END OF ANNEX